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Particulate Tubulin in Interphase and Metaphase Extracts of Oocytes of *Spisula*

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Abstract. In 1972 Weisenberg reported that surf clam oocytes contained a particulate and sedimentable pool of tubulin that could be isolated in buffers containing hexylene glycol. This "interphase particulate tubulin" (IPT) copurified with 10-20 μm granular spheres, which were identified as the "tubulin-containing structures" (TCS). Approximately one TCS per oocyte was isolated and the TCS disappeared after nuclear envelope breakdown. Weisenberg postulated that the TCS was comprised of a stored form of tubulin or a microtubule-assembly intermediate.

To characterize this intriguing form of stored tubulin, IPT was isolated in hexylene glycol-containing buffers as described by Weisenberg (1972, *J. Cell Biol.* 54, 266–278) and the amount of sedimentable tubulin was quantitated by immunoblotting during the first meiotic cell cycle. Approximately 10% of the total tubulin in *Spisula* oocytes sediments at g forces that are too small to pellet tubulin dimers or even single microtubules. Granular spheres, approximately 15 μm in diameter, are present in the sedimentable tubulin fractions. During the first cell cycle, the granular spheres disappear while the sedimentable tubulin levels gradually decrease. Although the disappearance of the spheres corresponds with the loss of sedimentable tubulin, the spheres do not contain tubulin. An initial centrifugation of the oocyte homogenates at 650g leaves most of the tubulin in the supernatant and the granular spheres in the pellet. The tubulin-containing fractions are composed of membranes and an amorphous unidentified material associated with short microtubules. Sedimentable tubulin is not detected in homogenates prepared at 0°C or in the absence of hexylene glycol, conditions that favor microtubule disassembly. It is likely that sedimentable tubulin is composed of hexylene glycol-induced polymers and not unique particulate structures that sequester tubulin. Finally, the granular spheres that contaminate the tubulin preparations are identified as nucleoli. They are morphologically identical to the nucleoli of the intact oocyte and they fluoresce brightly when stained with the Hoechst DNA dye 22358.

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Abbreviations: IPT—interphase particulate tubulin; TCS—tubulin-containing structure; MFNSW—millipore-filtered natural seawater; hexylene glycol—2-methyl-2,4-pentanediol; HGL—hexylene glycol-containing phosphate buffer; GV—germinal vesicle; PIPES—piperazine-N,N-bis(2-ethane-sulfonic acid); EGTA—ethylene glycol-bis(β-amino-ethyl ether) N,N,N′,N′-tetraacetic acid.

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Introduction

The regulation of microtubule length and spatial organization is important for diverse cellular processes such as axoplasmic transport and chromosome movements. Since microtubules are in a dynamic equilibrium with their tubulin subunits (Inoué, 1981; Mitchison and Kirschner, 1984), factors that perturb the free tubulin concentration can radically change microtubule performance (Mitchison and Kirschner, 1987; DeBrabander *et al.*, 1986; Avila 1990). In many cells, the polymer-monomer equilibrium is checked and balanced by regulating tubulin biosynthesis (Lefebvre and Rosenbaum, 1986; Cleveland *et al.*, 1988). Levels of unpolymerized tubulin subunits can censor their own synthesis as well as regulate microtubule assembly dynamics.

The mechanism for maintaining unassembled tubulin within the cytoplasm is not understood. *In vitro* studies have shown that a significant amount of unassembled tubulin is found in cellular fractions obtained by low speed centrifugation. In neuroblastoma cells, for example,

6–10% of the total tubulin is present in an insoluble, non-microtubular form (Hiller and Weber, 1978; Nath and Flavin, 1979; Olmsted, 1981). A membrane-associated or membrane-bound tubulin is also a common finding in neuronal cells and tissues (Stephens, 1986, 1990). Whether these fractions represent a unique unassembled form of tubulin is not established.

One of the most provocative studies of sedimentable tubulin was described by Weisenberg (1972). A particulate and sedimentable form of tubulin was found in surf clam oocytes and named "interphase-particulate tubulin" (IPT). Interphase-particulate tubulin was operationally defined as a colchicine-binding activity that sedimented at extremely low g forces and it was suggested that IPT is either a storage form of tubulin or a microtubule assembly intermediate. Spherical structures (10–20 μm in diameter) were identified as the particulate tubulin-containing structure or TCS. There was only one such TCS per oocyte. Moreover, the TCS disappeared and the level of IPT decreased during the first meiotic cell cycle. The tantalizing implication from these experiments was that an insoluble form of tubulin contained within a specific structure might regulate microtubule polymerization during the cell cycle. In this report, the presence and identity of the TCS and IPT is reexamined in order to characterize a potentially unique form of tubulin that may play an important role in microtubule dynamics during the embryonic cell cycle.

Figure 1. Identification of particulate tubulin extracts of oocytes of *Spisula* by Western blotting. The left panel illustrates the protein composition of the 2500g supernatant (S) and pellet (P) analyzed by SDS-PAGE and stained with Coomassie blue. Duplicate protein samples were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose. A monoclonal antibody (DM 1A) against α-tubulin was used to probe an immunoblot of the 2500g supernatant (S') and pellet (P). DM1A is a commercially available mouse monoclonal antibody prepared against chick brain α-tubulin (Blose *et al.*, 1984). The epitope for this antibody is located at the acidic C-terminal domain of the α-subunit (Serrano *et al.*, 1986; Breitling and Little, 1986). The position of α-tubulin is noted. Relative molecular mass standards are in kilodaltons.

Materials and Methods

Particulate tubulin isolation

Individuals of *Spisula solidissima* were provided by the Department of Marine Resources at the Marine Biological Laboratory (Woods Hole, MA). Ovaries were dissected and minced into Millipore-filtered (0.22 μm) natural seawater (MFNSW) and filtered through several layers of cheesecloth to obtain free oocytes. Oocytes were washed once in MFNSW and three times in Ca^{++}-free artificial seawater (Salmon, 1982). Oocytes were washed and kept at room temperature (21–22°C) for no longer than one hour. Oocytes were parthenogenetically activated by the addition of 14 ml of 0.5 M KCl in distilled water to a dilute suspension of oocytes (5 ml oocytes in 86 ml seawater) (Allen, 1953). Particulate tubulin was isolated at room temperature from an equal volume of oocytes at 0, 3, 6, 9, 12, 15, 18, and 21 min following activation. At each time point a small sample was immediately fixed in 1 ml of 75% ethanol and 25% acetic acid and later stained with lacto-orcein to visualize the chromosomes and accurately stage the cell cycle progression (Westendorf *et al.*, 1989).

Particulate tubulin was isolated from germinal vesicle stage oocytes and from parthenogenetically activated oocytes during the first meiotic cell cycle. One ml of packed oocytes was suspended at room temperature in ten volumes of HGL solution containing 1 M 2-methyl-2,4-pentanediol (hexylene glycol), and 0.01 *M* potassium phosphate at pH 6.2. The oocytes were homogenized with a Wheaton Dura-Grind Stainless Steel Dounce homogenizer with a 0.0005″ wall clearance. It was not necessary to remove or soften the vitelline envelop when using this homogenizer, so homogenates could be prepared rapidly at 3-min intervals. Homogenates were also prepared with a teflon pestle-glass homogenizer used by Weisenberg (1972); identical results were obtained with this instrument even though it was necessary to first soften the vitelline layer with glycerol before cell lysis (Rebhun and Sharpless, 1964). The homogenate was underlayed with a 5 ml cushion of 10% (w/v) sucrose in HGL and centrifuged at 4°C in a low-speed swinging bucket rotor at 5000rpm for 30 min (Beckman JS-13.1) (2500g at r_{av}). The 2500g

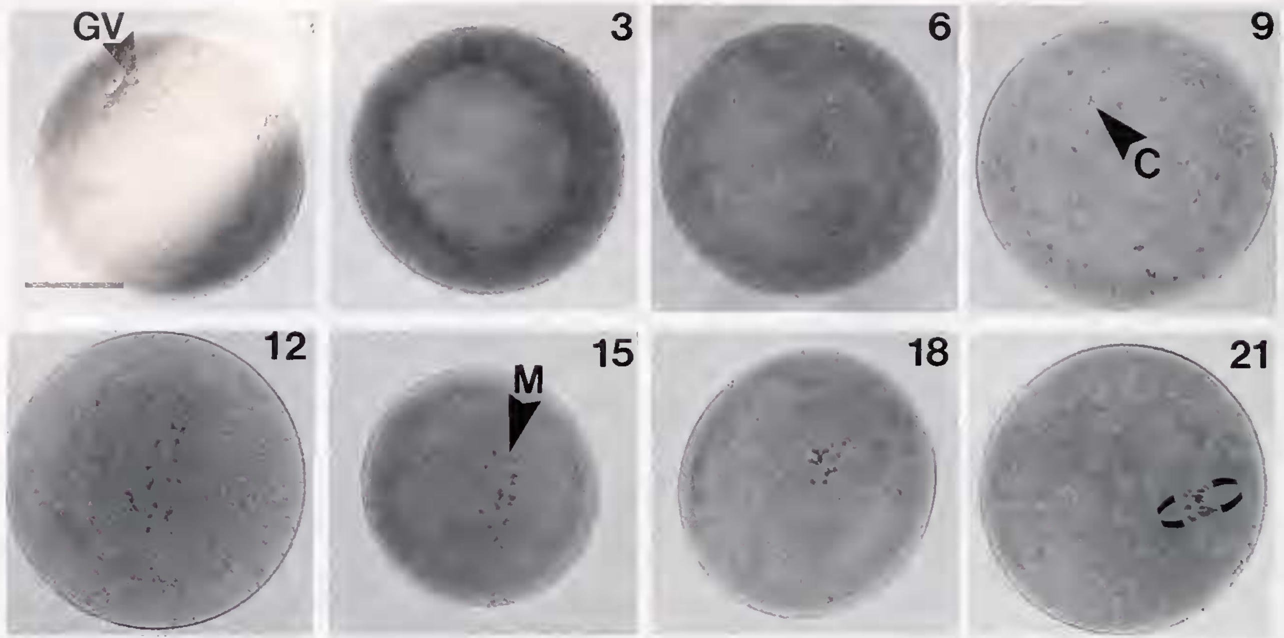

Figure 2. Oocyte development in *Spisula* following parthenogenetic activation visualized by brightfield light microscopy. Oocytes were fixed with ethanol-acetic acid and stained with lacto-orcein to visualize the chromosomes. The germinal vesicle (GV) present in the unactivated oocyte breaks down 9–10 min after activation. The chromosomes (C) are easily visualized after staining with lactoorcein as minute tetrads. The chromosomes congress towards the metaphase plate (M) as the first meiotic spindle forms in the center of the oocyte by 15′ post-activation. Chromosomes begin to separate as the first meiotic spindle (position indicated by brackets) moves to the cortex at 21′. The oocytes were flattened to different extents in these micrographs. Bar = 30 μm.

supernatant and sucrose cushion were removed, mixed together, and placed on ice. The 2500g pellet was resuspended stoichiometrically in 16 ml of ice cold HGL (volume equal to total homogenate volume plus sucrose cushion). Both supernatant and pellet fractions were frozen for later analysis.

Measurement of protein concentration

Protein concentrations were determined by the method of Lowry *et al.* (1951). Bovine serum albumin (BSA) was used as a protein standard.

Polyacrylamide gel electrophoresis

Proteins were analyzed on 0.75 mm SDS-polyacrylamide gels with the discontinuous buffer formulation of Laemmli (1970). The separating gel contained 8% acrylamide and 4 *M* urea. Gels were stained for protein with Coomassie blue in a concentrated methanol/acetic acid solution (Sambrook *et al.*, 1989).

Immunoblotting

For immunoblot analysis, unstained SDS-polyacrylamide gels were electrophoretically transferred to nitrocellulose (BA-45, Schleicher and Schuell, Keene, NH) as described by Towbin *et al.* (1979). The nitrocellulose blot was air-dried for 1–3 h and then blocked with Tris-buffered saline (TBS) (10 m*M* Tris-HCl, pH 7.4, 0.15 *M* NaCl) containing 5% (w/v) Carnation's non-fat dry milk for 45–60 min at room temperature. After blocking, the blot was incubated for 18–24 h (4°C) with a monoclonal antibody specific for α-tubulin (DM1A) (ICN Immunobiologicals, Lisle, IL) diluted 1:500 in TBS with 5% milk. After washing (4 times, 10 min each in TBS), the blot was blocked again for 15 min in TBS with 5% milk then incubated in a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Hyclone Laboratories, Logan, UT) in TBS-milk for 2–3 h at room temperature. The blot was washed again in TBS (4 times, 10 min) and incubated 5–15 min in freshly prepared developer (20 ml TBS, 4 ml of 3 mg/ml 4-chloro-1-napthol in methanol, 20 μl of 30% hydrogen peroxide) (Hawkes *et al.*, 1982). The blots were rinsed in deionized water, air-dried, and photographed.

Light and electron microscopy

Preparations were examined with a Nikon Optiphot equipped with phase and fluorescence light optics. Images

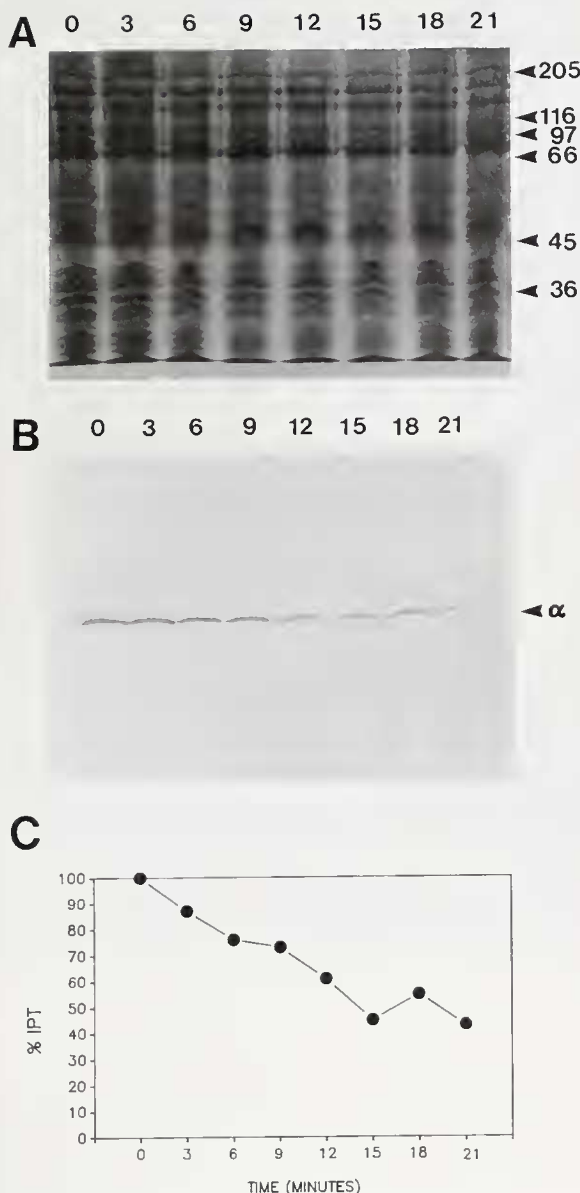

Figure 3. Quantitation of IPT during the first meiotic cell cycle in extracts of oocytes of *Spisula*. Particulate tubulin fractions were isolated from equal volumes of oocytes at 3′ intervals following parthenogenetic activation. An equal volume of each particulate tubulin fraction was separated by SDS-PAGE and stained with Coomassie blue (A). Identical samples were blotted to nitrocellulose and probed with a monoclonal antibody against α-tubulin (B). The blots were scanned with an Apple OneScanner interfaced to an Apple IIcx computer and analyzed with the public domain software program IMAGE. The amount of sedimentable tubulin obtained at each interval is expressed as a percent of the total interphase particulate tubulin (IPT) detected in the oocyte (C).

were recorded on TMAX 100 and 400 film developed in TMAX developer. For electron microscopy, samples were negatively stained with uranyl acetate on glow discharged, carbon-coated formvar grids.

Results

Interphase particulate tubulin (IPT) and the tubulin-containing structure (TCS)

A significant fraction (>10%) of the total detectable tubulin in surf clam oocytes sediments at very low centrifugal forces (Fig. 1). This tubulin corresponds to the interphase particulate tubulin (IPT) described by Weisenberg (1972). Following oocyte activation, the level of particulate tubulin decreases steadily during the time course of nuclear envelop breakdown and formation of the first meiotic spindle (Figs. 2, 3). The decrease in sedimentable tubulin following parthenogenesis suggests that the interphase particulate tubulin (IPT) may be a storage form of tubulin that becomes solubilized.

Tubulin dimers (Mr 100,000) alone or single microtubules cannot pellet at 2500g's. There are, however, large 15 μm spherical particles present in unactivated oocyte homogenates that might be associated with the particulate tubulin (Fig. 4); these particles are conspicuously absent from homogenates prepared 10 min after parthenogenetic activation. In addition, these large particles pellet with the particulate tubulin (Fig. 5). After centrifugation through sucrose, the particles frequently take on an ovoid profile and become more granular in appearance. These large particles are actually composed of two tandemly arranged spheres. The smaller 3–5 μm spheres are frequently detached from the larger 12–13 μm spheres (Fig. 5C–F). These spherical particles bear a striking resemblance to the nucleolus and nucleolinus of the intact germinal vesicle stage oocytes (Fig. 5A). To confirm their identity, these structures were incubated with Hoechst 33258 and examined by fluorescence light microscopy (Fig. 6). Both the large and small particle fluoresce brightly revealing their DNA content.

The nucleolus is not a tubulin-containing structure

To determine if any sedimentable tubulin was associated with the nucleoli, the nucleoli were separated from the tubulin by centrifugation. Oocytes were homogenized in HGL, underlayed with a sucrose cushion and centrifuged at 650g (2500 rpm) for 5 min in a clinical centrifuge. Four fractions were obtained—the uppermost supernatant S1, the sucrose interface S2, the sucrose cushion S3, and the pellet (Fig. 7A). Each fraction was resuspended stoichiometrically in 11 ml of HGL (starting volume of homogenate) and small aliquots were examined by phase microscopy (Fig. 7C). Under these centrifugation conditions the nucleoli pellet to the bottom of the tube and are not detected in any of the supernatant fractions.

Sedimentable tubulin is detected in all fractions (Fig. 7B). Each 11 ml fraction was underlayed with 10% sucrose

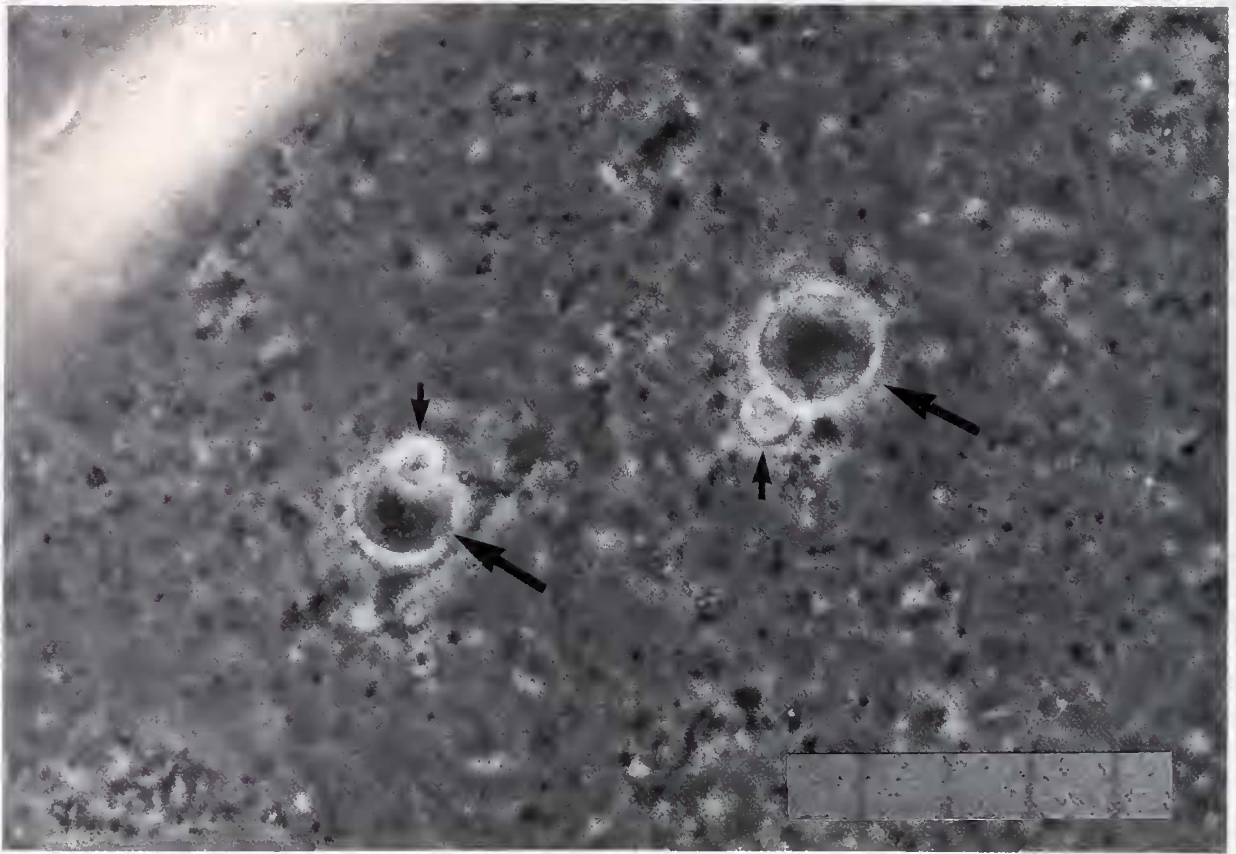

Figure 4. Homogenates of unactivated surf clam oocytes contain large, tandemly arranged, globular structures. In phase microscopy, a large, phase-dark sphere (large arrows) is associated with a small, round, phase-light particle (small arrows). These tandemly arranged particles are frequently associated with a phase-dark membrane-like structure (see particle on the left). A magnification bar with 10 μm spacing is shown.

in HGL and centrifuged at 2500g to pellet the particulate tubulin. An equal volume of each resuspended pellet was analyzed and compared by SDS-PAGE and immunoblotting (Fig. 7B). Each 650g fraction—S1, S2, S3, and P—contained tubulin that sedimented at 2500g. The largest amount of particulate tubulin was found in the fractions farthest away from the nucleoli (Table I). This does not rule out the possibility that a small amount of sedimentable tubulin still might be associated with the nucleoli. However, no nucleolar tubulin was detected by immunofluorescence with the DM 1A antibody. The levels of nucleolar fluorescence were indistinguishable from the controls in which no first antibody was added.

To identify the source of the particulate tubulin, a small sample of each resuspended pellet (2500g) was negatively stained with uranyl acetate for transmission electron microscopy (Fig. 7D). The bottom three fractions contain numerous short microtubules which are associated with membranes and densely staining, amorphous aggregates. No microtubules are detected in the uppermost fraction (S1), which also contained numerous membranes, granular aggregates, and the largest fraction of particulate tubulin.

The abundance of membranes in all fractions suggested that the sedimentable tubulin might be membrane-bound. The amount of IPT that was detergent-soluble was examined before and 5 min after parthenogenetic activation. Homogenates were prepared in HGL in the presence and absence of 0.5% Triton-X-100 and the amount of tubulin in the 2500g pellets was compared by immunoblotting. Inclusion of this non-ionic detergent has no effect on the amount of sedimentable tubulin detected before or after activation, indicating that the sedimentation of this tubulin is not dependent upon membrane integrity.

The presence of microtubules in three of the four fractions suggests that microtubule assembly may have taken place in the homogenates prepared *in vitro*. The hexylene glycol used in this and the previous study (Weisenberg, 1972) may have promoted the assembly of microtubules and their association with the denser and more massive

Figure 5. Phase microscopy of particulate tubulin fractions in extracts of oocytes of *Spisula.* Unactivated oocytes (A), were homogenized and centrifuged as described in the Materials and Methods to yield a 2500 g supernatant (B) and 2500g pellet that contains the interphase particulate tubulin (C–F). Tandemly arranged large spheres (large arrows) and small particles (small arrows) are absent from the supernatant fractions. After centrifugation, the small particle is frequently lost and only the larger sphere is observed. Membrane-like (M) structures are frequently associated with the larger sphere. Compare the morphology of the tandemly arranged particles to the nucleolus (N) and nucleolinus (L) of the intact oocyte (5A). Within the germinal vesicle (GV) lie a tandemly arranged structure indistinguishable from the structures in the 2500g pellet. Bar = 20 μm.

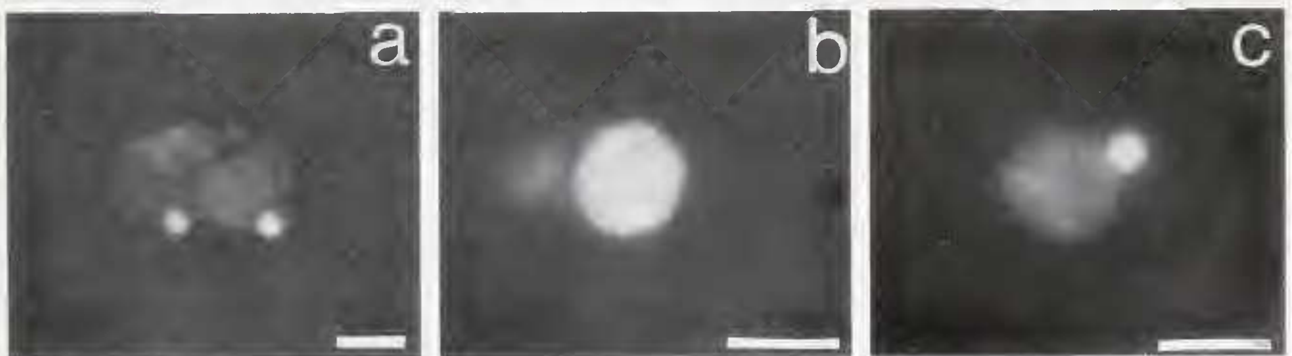

Figure 6. Hoechst DNA staining of the 2500g pellet fraction. Pellets were resuspended in HGL, incubated for 10 min on poly-L-lysine coated coverslips, rinsed twice with HGL and finally with HGL containing 2 μg/ml Hoechst 22358. The magnification bar is 10 μm.

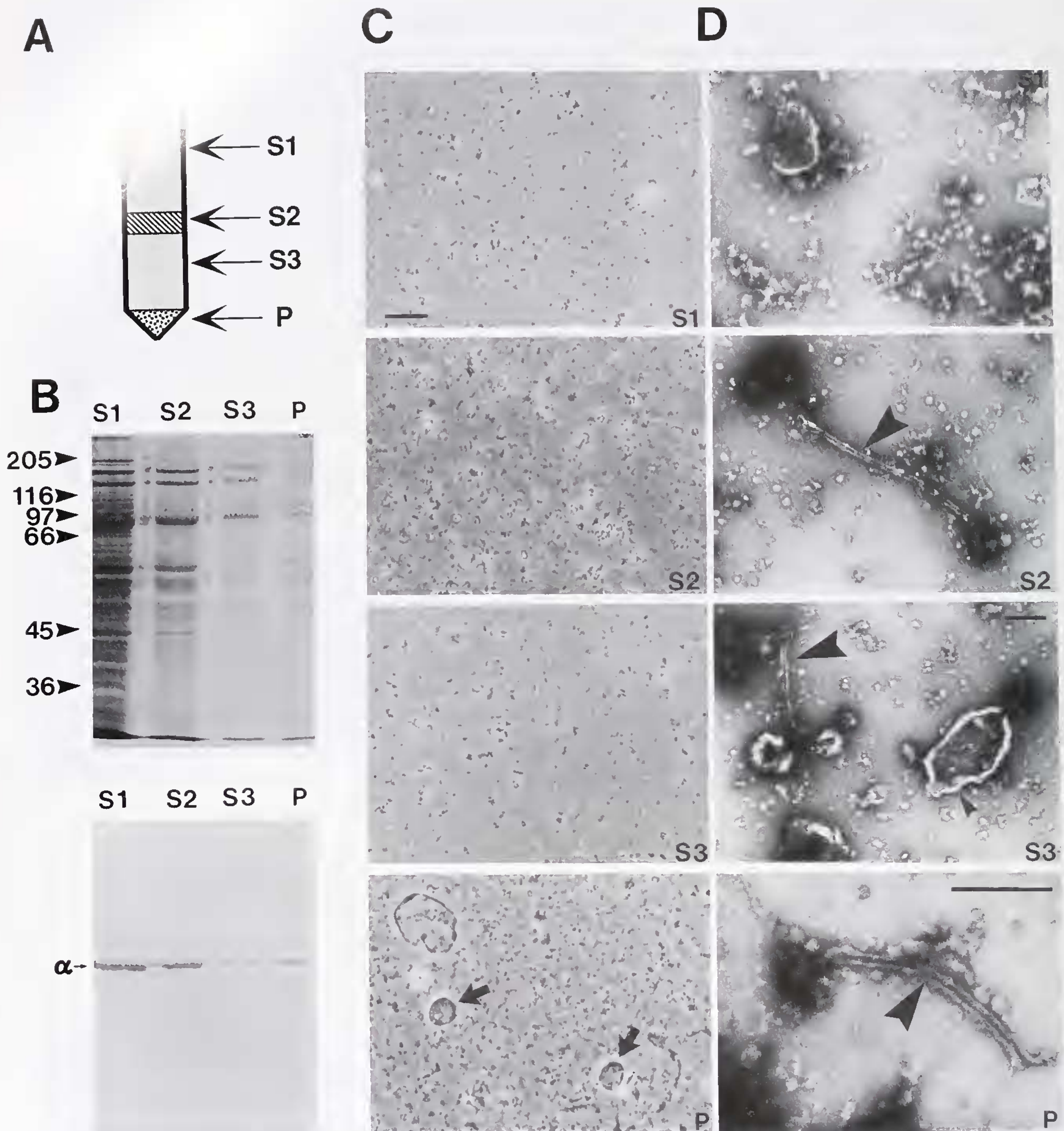

Figure 7. Separation of the nucleoli from the particulate tubulin-containing fractions in extracts of oocytes of *Spisula* by low speed centrifugation. Panel A: Diagram illustrating the four fractions obtained after a 650g centrifugation (see Results). Each 650g fraction was diluted in HGL and examined by phase microscopy (Panel C). The nucleoli are only detected in 650g pellet (P). Each 650g fraction was centrifuged again to obtain a 2500g pellet. SDS-PAGE analysis of each 2500g pellet is shown in Panel B (top). Identically prepared samples were blotted to nitrocellulose and probed with the DM1A antibody against α-tubulin. Each 650g fraction contains a significant amount of tubulin that sediments at 2500g. Each 2500g pellet was resuspended in HGL and negatively stained with uranyl acetate for electron microscopy (Panel D). Membranes (small arrowheads) are present in every fraction while microtubules were detected in the three heaviest fractions—S2, S3, and P. Magnification bar is equal to 20 μm in panel C and 0.25 μm in panel D.

Table I

Amount of particulate tubulin in each 650g fraction expressed as a percentage of the total interphase particulate tubulin (IPT) found in all four fractions in extracts of oocytes of Spisula

Fraction	% Total IPT
S1	47.2
S2	33.3
S3	5.4
P	14.1

cytoplasmic aggregates and organelles. To examine this possibility, experiments were carried out under conditions that favored microtubule disassembly. Homogenates were prepared in either ice cold HGL or in phosphate buffers at room temperature in the absence of hexylene glycol. There is no detectable tubulin in the 2500g pellets under either of these conditions. Finally, homogenates were prepared at pH 6.6, 6.9, and 7.2 in ice-cold microtubule-reassembly buffer that contained PIPES, $MgSO_4$, EGTA, GTP, DTT, protease inhibitors, and no hexylene glycol. Several pH values were tested, because it was shown recently that microtubule assembly in surf clam oocyte extracts is favored at alkaline pH (Suprenant 1989; 1991). Under these isolation conditions, all the tubulin is detected in the supernatant fluids (Fig. 8). There is no sign of interphase particulate tubulin on these heavily overloaded gels. These results indicate that the microtubules found in the particulate tubulin preparation are either stabilized or induced to assemble by the hexylene glycol.

Discussion

Fertilization of surf clam oocytes results in a major reorganization of the cytoplasm that ultimately results in syngamy and the first mitotic cleavage division (Allen, 1953; Rebhun, 1959; Longo and Anderson, 1970a, b; Dan and Itoh, 1984). During the 75 min following fertilization, microtubules of two meiotic and one mitotic apparatus are assembled and disassembled. The regulatory events that are responsible for these transitions in microtubule polymerization are not known. The unfertilized clam oocyte does contain up to 30 μM tubulin (Burnside *et al.*, 1973; Suprenant, 1991), none of which appears to be assembled into microtubules (Kuriyama *et al.*, 1986). Weisenberg (1972) proposed that this unpolymerized tubulin was sequestered in discrete spherical, tubulin-containing structures (TCS).

In this study, the particulate and sedimentable pool of tubulin, first described by Weisenberg (1972) as "interphase-particulate tubulin" or IPT, was isolated from surf clam oocytes. Every attempt was made to duplicate the isolation conditions described by Weisenberg. The only substantive change was the assay used to identify and quantitate the IPT. Instead of a colchicine-binding assay, tubulin was solubilized, separated from other oocyte proteins by electrophoresis, and quantitated by Western blotting with a monoclonal antibody, DM 1A, generated against chick brain microtubules (Blose *et al.*, 1984). An immunoassay is more advantageous than a colchicine-binding assay because it is very sensitive and highly specific for tubulin even in a total cellular homogenate and it avoids the thermal inactivation or decay of the colchicine-binding reaction.

This study and Weisenberg's (1972), have shown that over 10% of the total tubulin in surf clam homogenates is "particulate" and sediments at very low g forces. Because the amount of IPT decreases following activation, Weisenberg (1972) suggested the exciting possibility that IPT was a stored form of tubulin or microtubule-assembly intermediate. The results from this study indicate that this hypothesis is probably not true because both the microtubule-associated and aggregated forms of IPT are dependent upon hexylene glycol. High concentrations of solvents (1–4 M) such as glycerol (Lee and Timasheff, 1977), DMSO (Himes *et al.*, 1976), polyethylene glycol (Herzog and Weber, 1978), and hexylene glycol (Kane, 1962; Rebhun and Sharpless, 1974) can promote the polymerization of tubulin and stabilize microtubules against disassembly.

It is likely that the decrease in IPT following oocyte activation reflects the *in vitro* cytoplasmic assembly conditions. For example, recent studies have examined nucleated-microtubule growth at defined cell cycle states in *Xenopus* cell-free extracts (Gard and Kirschner, 1987a, b; Belmont *et al.*, 1990; Verde *et al.*, 1990; Gotoh *et al.*, 1991). Visualization of microtubules in real-time and in fixed preparations reveals that microtubules assembled in mitotic extracts are shorter and more dynamic than those in interphase extracts. If microtubules in surf clam extracts behave similarly, the transition from interphase to mitosis would be accompanied by an increase in microtubule turnover and a decrease in the steady state mass of assembled polymer.

Similar to the previous report (Weisenberg, 1972), unactivated surf clam homogenates contain large granular particles as well as a form of tubulin that sediments at low g forces. In this study, the granular particle is identified as the nucleolus, not the tubulin-containing structure. The nucleolus is not the source of the interphase particulate tubulin since it can be separated from the bulk of the sedimentable tubulin and tubulin cannot be detected within the nucleolus by immunofluorescence. The granular particle is identified as the nucleolus because it: (1) is present at only one copy per cell, (2) is morphologically

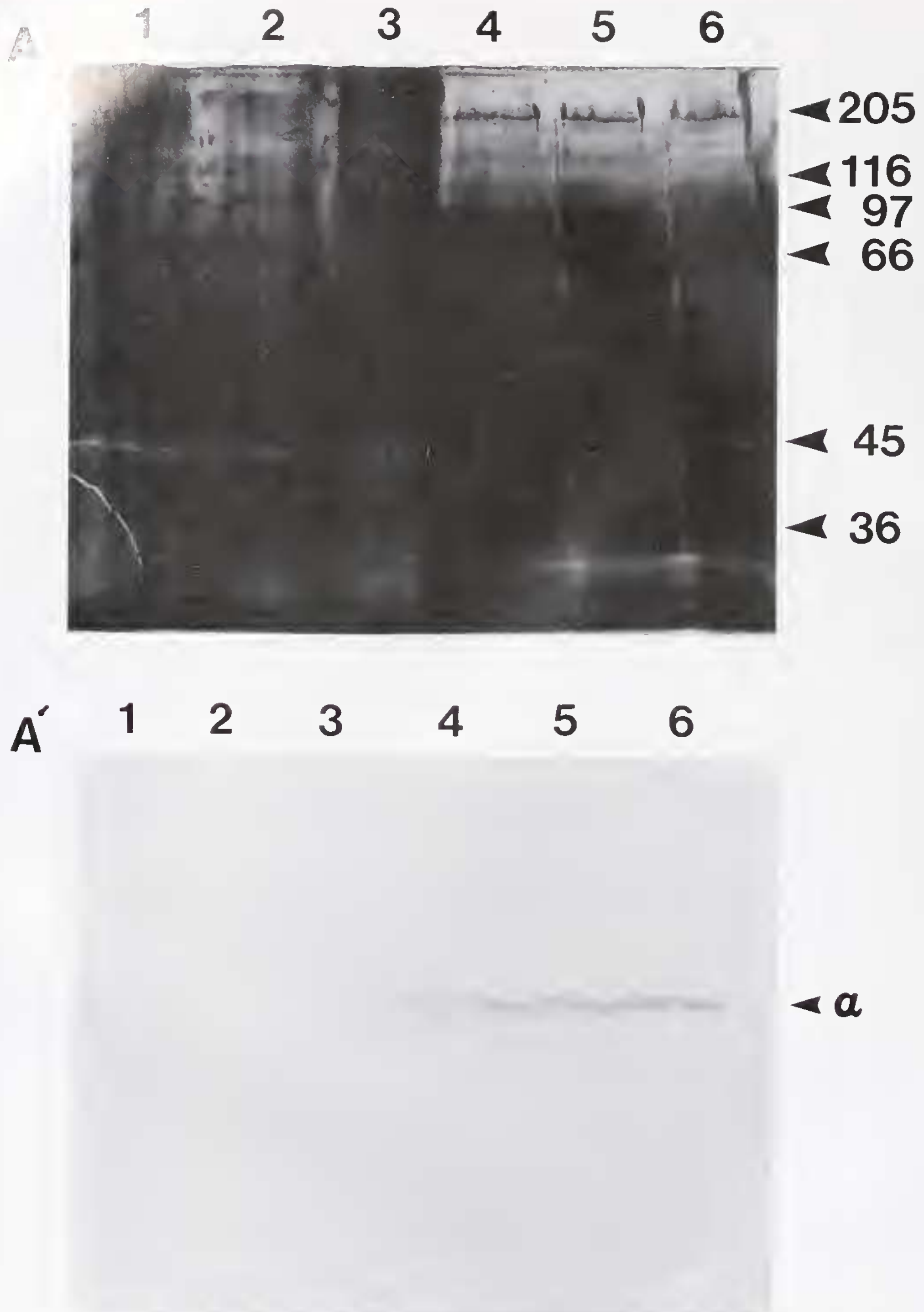

Figure 8. Sedimentable tubulin was absent from homogenates prepared in a microtubule assembly buffer without hexylene glycol. Surf clam oocytes were homogenized in a buffer containing 100 m*M* K-PIPES, 4 m*M* EGTA, 1 m*M* $MgSO_4$, 1 m*M* DTT, 0.2 m*M* PMSF, 10 μg/ml leupeptin, and 1 m*M* GTP, at either pH 6.6, 6.9, or 7.2. Homogenates were centrifuged at 2500g through a 10% sucrose cushion prepared at the appropriate pH. Proteins in the supernatant and pellet were separated by SDS-PAGE and electroblotted to nitrocellulose. Panel A is Western blot stained for total protein with India ink (Hughes *et al.*, 1988). Panel A′ is the corresponding immunoblot with the DM1A anti-α-tubulin antibody. Lanes 1, 2, and 3 are the 2500g pellets from the pH 6.6, 6.9, and 7.2 homogenates and Lanes 4, 5, and 6 are the corresponding supernates at pH 6.6, 6.9, and 7.2. Note the absence of pelletable tubulin in all three pellets.

identical to the nucleolus in the intact cell, (3) stains intensely with Hoechst, and (4) disappears at the time of breakdown of the nuclear envelop.

Finally, it is important to note that a significant fraction of the particulate tubulin is not associated with any detectable microtubules. Whether this particulate tubulin represents a functional form of tubulin that is able to form microtubules has not been established. In this study, all attempts to reassemble microtubules from the aggregated particulate tubulin fractions were unsuccessful. As the ionic strength of the oocyte cytoplasm is normally quite high, it is likely that some of this tubulin is denatured by the low ionic strength potassium phosphate buffer used throughout most of this study. Particulate tubulin was not detected in a higher ionic strength, PIPES-buffered microtubule-assembly solution, nor was it detected in Buffer T (Westendorf *et al.*, 1989). Buffer T is formulated to mimic the known constituents of molluscan cytoplasm and contains 0.3 *M* glycine, 0.12 *M* K-gluconate, 0.1 *M* taurine, 40 m*M* NaCl, 10 m*M* EGTA, 2.5 m*M* $MgCl_2$, and 0.1 *M* HEPES at pH 7.2. Thus, it appears that a large fraction of the "interphase-particulate tubulin" is artifactual or at least is operationally dependent upon the isolation conditions.

Acknowledgments

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